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Award Number: DAMD17-03-1-0488

TITLE: Bioavailability of TGF-Beta in Breast Cancer

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REPORT DATE: July 2007

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

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1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
01-07-2007	Annual Summary	15 Jul 2006 – 14 Jul 2007

4. TITLE AND SUBTITLE

Bioavailability of TGF-Beta in Breast Cancer

5a. CONTRACT NUMBER

5b. GRANT NUMBER
DAMD17-03-1-0488

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of California
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Berkeley, CA 94720

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
The Transforming Growth Factor beta (TGF-b) superfamily includes three isoforms designated TGF-b1, b2 and b3. All three isoforms are secreted as latent complex where the TGF-b cytokine is non-covalently associated with an isoform specific latency-associated peptide (LAP). Mature cytokine binds cell surface receptors only after release from its LAP making extracellular activation a critical regulatory point for TGF-b bioavailability. Proposed activation mechanisms include proteolysis and conformational changes. Previous work from our laboratory showed that latent TGF-b1 (LTGF-b1) is efficiently activated upon exposure to reactive oxygen species (ROS). ROS activation is restricted to the LTGF-b1 isoform. Because of the amino acid sequence differences between the three LAPs, we postulate that the specificity of this activation mechanism lies within the LAP. Furthermore, we hypothesize that the presence of a metal in the latent complex could provide a redox active center for this process. Redox mediated activation provides a novel mechanism for TGF-b participation in tissues undergoing oxidative stress. Moreover, this would allow TGF-b1 to act both as a sensor of oxidative stress within tissues as well as a transducer of that signal by binding to its cellular receptors.

15. SUBJECT TERMS Transforming Growth Factor-beta, activation, reactive oxygen species, oxidation, tumorigenesis, metal binding, isoform specificity, X-ray crystallography

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	16	19b. TELEPHONE NUMBER *(include area code)*

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Introduction

The Transforming Growth Factor beta (TGF-β) superfamily includes three isoforms designated TGF-β1, β2 and β3. All three isoforms are secreted as latent complex where the TGF-β cytokine is non-covalently associated with an isoform specific latency-associated peptide (LAP). Mature cytokine binds cell surface receptors only after release from its LAP making extracellular activation a critical regulatory point for TGF-β bioavailability. Proposed activation mechanisms include proteolysis and conformational changes. Previous work from our laboratory showed that latent TGF-β1 (LTGF-β1) is efficiently activated upon exposure to reactive oxygen species (ROS).

ROS activation is restricted to the LTGF-β1 isoform. Because of the amino acid sequence differences between the three LAPs, we postulate that the specificity of this activation mechanism lies within the LAP. Furthermore, we hypothesize that the presence of a metal in the latent complex could provide a redox active center for this process. Redox mediated activation provides a novel mechanism for TGF-β participation in tissues undergoing oxidative stress. Moreover, this would allow TGF-β1 to act both as a sensor of oxidative stress within tissues as well as a transducer of that signal by binding to its cellular receptors.

Body

Specific Aims

1. Characterize the interaction between reactive oxygen and latent TGF-β
2. To identify and localize the redox-metal center within the latent TGF-β
    a. We have begun to optimize a method based on chemiluminescence and colorimetric detection assays to identify the presence of metal cofactors.
    b. We initiated the generation of an immortalized TGF-β null mouse embryonic fibroblast (MEF) line that will be used in transfection studies aimed at expressing either wild type LTGF-β1 or LTGF-β1 mutants sensitive or not to ROS.
3. Determine the three-dimensional structure of latent TGF-β

Progress

Because Aim #1 is completed, to date, the efforts have been centered into Aim #2. We started to optimize a method to identify the presence of transition metals in the latent TGF-β. The main advantage of this chemiluminescent method is the low amount of protein needed, on a microgram scale. About Aim #3, the crystal structure of LTGF-β is being determined by Dr. Peter Walian.

Aim 2. To identify and localize the redox-metal center within the latent TGF-β (LTGF-β)

Using metal ion-catalyzed ascorbate oxidation to generate reactive oxygen species (ROS) we have observed that LTGF-β is activated with high efficiency by ROS (Barcellos-Hoff and Dix 1996). The presence of a transition metal bound adventitiously or specifically to LAP could lead to specific oxidation of certain aminoacids. According to the obtained data, the presence of a transition metal related to LAP has been postulated. Recently, we obtained more data that give support to the presence of a transition metal in the latent complex. As shown in our report last year, we have demonstrated the specific activation of LTGF-β1 by ROS. More specifically, methionine 253 of the latent associate peptide β1 (LAP-β1) is a direct target for ROS. Also, we have shown that the oxidation mediated by ROS is reversible without denaturing the LAP (Jobling et al. 2006). These data define LTGF-β1 as a redox switch and support a potential role for transition metals activating the latent complex.

The standard methods to determine the presence of metals in proteins, like atomic absorption spectrometry, inductively coupled plasma combined with atomic emission spectroscopy (ICP-AES) or mass spectrometry (ICP-MS), or synchrotron techniques such as x-ray fluorescence spectrometry or extended x-ray absorption fine structure (EXAFS), use high amounts of protein. These tests are impracticable in the case of TGFβ which is not easy or cheap to synthesize and purify in the quantities required. Therefore, we optimized a new chemiluminescent and colorimetric method described by Hogbom et al (2005) which uses low concentration of proteins to determine the presence of transition metals.

This method consists of two consecutive tests; the first test is based on the catalytic reaction of some transition metals with luminol producing chemiluminescence and the second colorimetric test uses 4-(2pyridylazo)resorcinol (PAR) to form colored complexes with metals. Three measurements are done, two chemiluminescence assays, following addition of luminol and PAR, and finally a colorimetric assay one hour after the PAR addition. All tests are done in 96

well plates using a total volume of protein solution of 8µl with an optimum concentration of 5µg/µl.

To establish baselines, we tested several metallic salts (Iron (II) chloride, Cupper (II) chloride, Cobalt (II) chloride, Manganese (II) chloride and Nickel (II) chloride) in order to obtain a specific pattern of their behavior with both luminol and PAR reactions. All salts were used at 0.5mM which is equivalent to 4nmol per each well of a 96 well plate. Serial dilution was done to test 4, 2, 1 and 0.5 nmol. The results obtained (see graph 1 and table 1) show that a minimum working concentration of 1 nmol metal ion is required for chemiluminescent assays and at least 2 nmol for colorimetric assays. After the addition of luminol, iron, cupper and cobalt showed luminescence (see table 1 for results). After PAR addition, iron increased in luminescence whereas luminescence of cupper decreased and that of cobalt showed similar values. Manganese started to be luminescent after addition of PAR, while nickel never showed chemiluminescence. For the colorimetric assay, iron was always negative with a yellowish color similar to the negative control, whereas all the other metal solutions showed a positive reddish coloration.

We followed this standardization with several metalloproteinases to further test the luminescent and colorimetric assay. The proteins used were: super oxide dismutase, carbonic anhydrase, cytochrome-c and hemoglobin. A first assay was done following the optimum protein concentration of 5µg/ml given in the study made by Hogbom et al (2005). The results showed the presence of the correspondent metals for each metalloprotein, cupper for superoxide dismutase, zinc for carbonic anhydrase and iron for cytochrome-c and hemoglobin (see graph 2 and table 2). The same concentration of diverse metalloproteins contains different levels of ions because metalloproteins differ in molecular weight and in number of metal ions they bind. For a better control over the results on the test, we adjusted the concentration of each protein and metal to 1 nmol and to 0.1 nmol. However, our data showed that 1 nmol of metal ion per well was necessary to run both luminescent and colorimetric tests, whereas 0.1 nmol was only sufficient to run the luminescent but not the colorimetric test (see graph 3 and table 3).

It is difficult and expensive to obtain large quantities of LTGF-β, which motivated our use and optimization of both chemiluminescent and colorimetric tests. The protein concentration must be adjusted to obtain optimal results with the minimum expenses of LTGF-β. We therefore calculated the minimum amount of LTGF-β necessary for the assays according to its molecular weight (110KDa) and assuming one metal ion bound per molecule. The minimum amount of 1nmol of metal ion per well necessary as determined in the above standardization assays translates into 100µg of LTGF-β per well (8µl), which corresponds to a concentration of 12.5 µg/µl.

This is still a very high concentration of LTGF-β especially because it is biologically functional on the order of picomolar concentrations. It is very difficult to obtain by synthesis or commercially such high quantities of LTGF-β, even more for all three different isoforms. We have presently available enough quantities only for LTGF-β2, which was previously expressed and purified to test crystallization conditions. Because the maximum concentration available in our stock of LTGF-β2 is 1.7 µg/ml, we had to concentrate the protein using a centrifugal system. In parallel to our chemiluminescent and colorimetric tests, we used the standard bioassay method to check the current activity of the LTGF-β2 of both stock and concentrated samples. The bioassay test demonstrated that all samples of LTGF-β2 were completely active, which means that LAP and TGF-β were not forming a complex. The lack of interaction between LAP, were the metal ion is expected to be located, and TGF-β could explain the negative results with both luminol and PAR tests showing no presence of any metal (graph 4 and table 4). In order to investigate further

determination of transition metal binding to LTGF-β, we are looking for different sources to obtain the necessary amount of protein to run the analysis.

To further study the activation of LTGF-β1 by ROS, we will transfect immortalized wild type or TGFβ-null MEF with cDNAs encoding wild type LTGF-β1 or a mutant LTGF-β1 bearing a point mutation that substitutes methionine for alanine at position 253 (Jobling et al. 2006). We have shown that this M253A mutation leads to loss of response of LTGF-β1 to ROS.

Because transfection efficiency is very low for primary fibroblasts and because stable transfection is a long process, we propose to establish immortalized MEF cell lines prior to transfection. Primary MEF grow rapidly and are healthy until the 3rd passage, then they grow slowly and die in large number due to senescence. However, MEF cultures maintained for more than 20 passages, around 40 to 50 days, undergo spontaneous immortalization (Parrinello, 2003). We will follow a recently optimized immortalization procedure (Jianming Xu, 2005). The first step, currently in progress, consists of maintaining in culture MEFs isolated from wild type and TGF-β null mouse embryos until they reach immortalization. Once immortalized, these two MEF cell lines will be expanded then subjected to transfection.

Since stable transfection will require the use of the antibiotic neomycin (G418) as selection reagent, it is necessary to establish a cell death curve for MEFs in order to determine the minimal concentration of antibiotic necessary to kill non transfected cells. This minimal concentration of G418 will be added to the cell culture medium 48h post transfection in order to select for stably transfected cells. Three mutant versions of LTGFβ-1, bearing methionine → alanine substitutions at three different positions (M112A, M132A or M253A, respectively) have been cloned into the mammalian cell expression vector pCDNA3. Because the pCDNA3 vector is not suitable for retroviral gene transfer technology, cells will be transfected by lipofection using lipofectamine 2000 (Invitrogen) using the manufacturer's guidelines. Alternatively, other lipofection agents will be tested. DNAs will be linearized prior to transfection to ensure genomic integration.

After 4-6 weeks of selection with G418, MEF stably transfected with wild type or LTGFβ-1 mutants will be switched to serum-free conditioned medium shortly (24-48h) before performing LTGF-β1 activation assay. Media from MEF cultures will be collected and treated with ascorbic acid and Fe(III) to determine ROS activation. Medium subjected to heat, a treatment known to result in LTGFβ-1 activation, will be used as a positive control . According to our previous in vitro studies using the mutants M112A, M132A and M253A (Jobling et al. 2006), we anticipate that only cells expressing the M253A mutant will produce a LTGF-β1 resistant to ROS activation.

Aim 3. To determine the three-dimensional structure of latent TGF-β

Prior collaborations with Dr. Peter Walian here at LBNL, have not yielded conditions for crystallization of LTGF-β.

Key Research Accomplishments

- Optimization of a new method to determine the presence of a transition metal in proteins using relatively small amounts of protein, in a nanomolar scale.
- Development of immortalized wild type and TGF-β null MEF cell lines. Once immortalized, these cell lines will be transfected with cDNAs encoding wild type or three mutant versions of LTGFβ-1 (M112A, M132A, M253A).

Conclusions

We propose a simpler method than the traditional ones as atomic abortion spectrometry, ICP-AES, ICP-MS or EXAFS, to determine the presence of a transition metal in LTGF-β. Unfortunately, we don't have yet the necessary amount of protein required to run a determinative analysis, but actually we are looking for different sources to get the necessary amount of protein. The expression of LTGF-β1 mutant constructs into MEF will provide further mechanistic understanding of ROS-mediated TGF-β activation.

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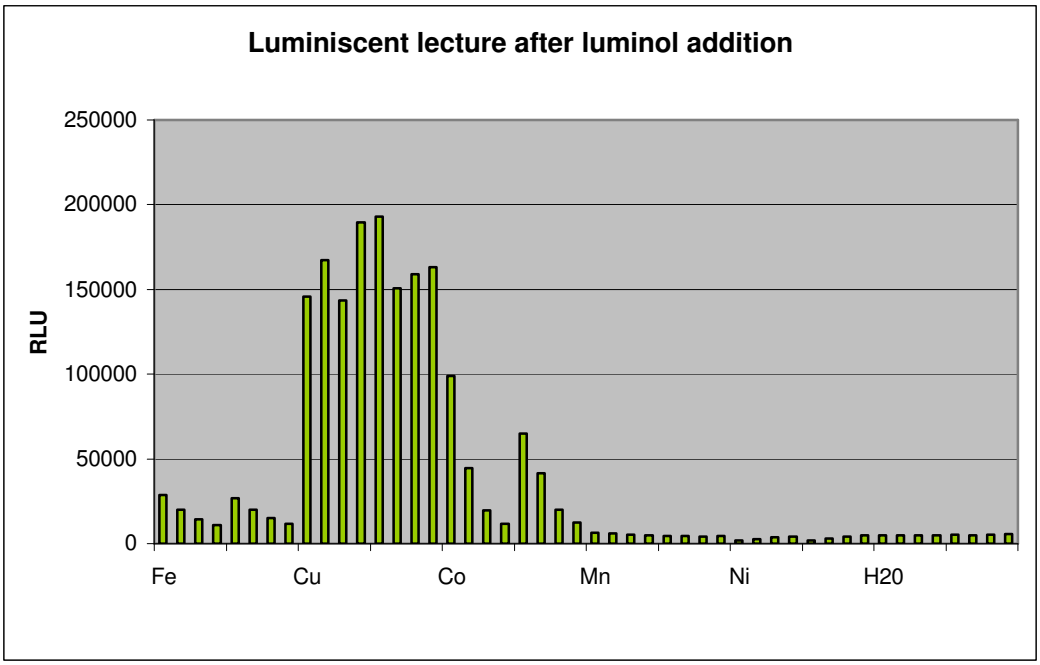
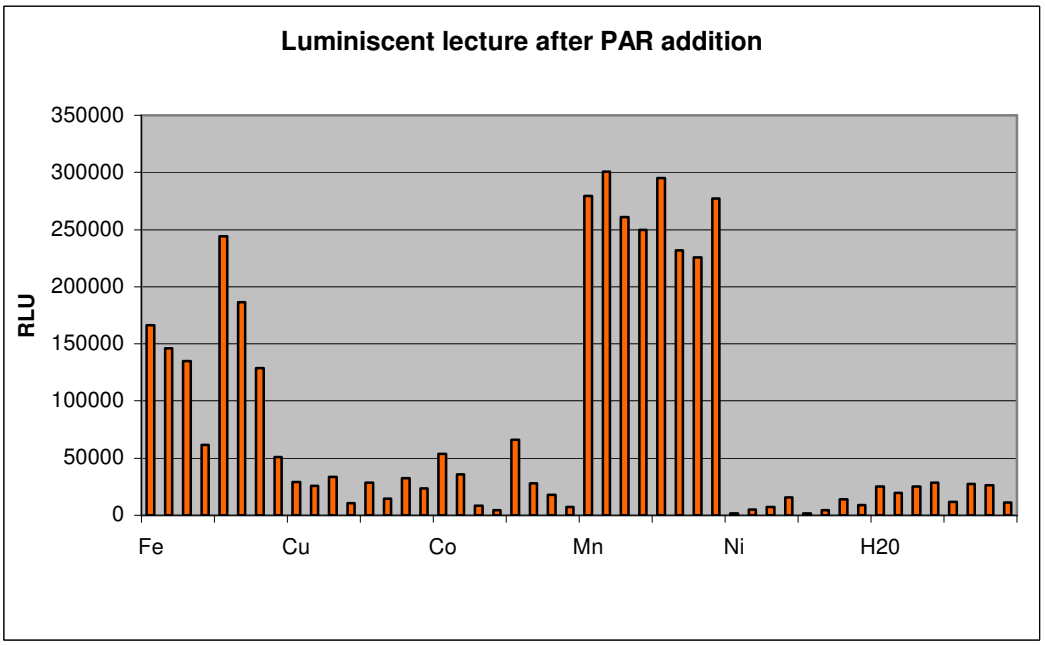
Supporting data

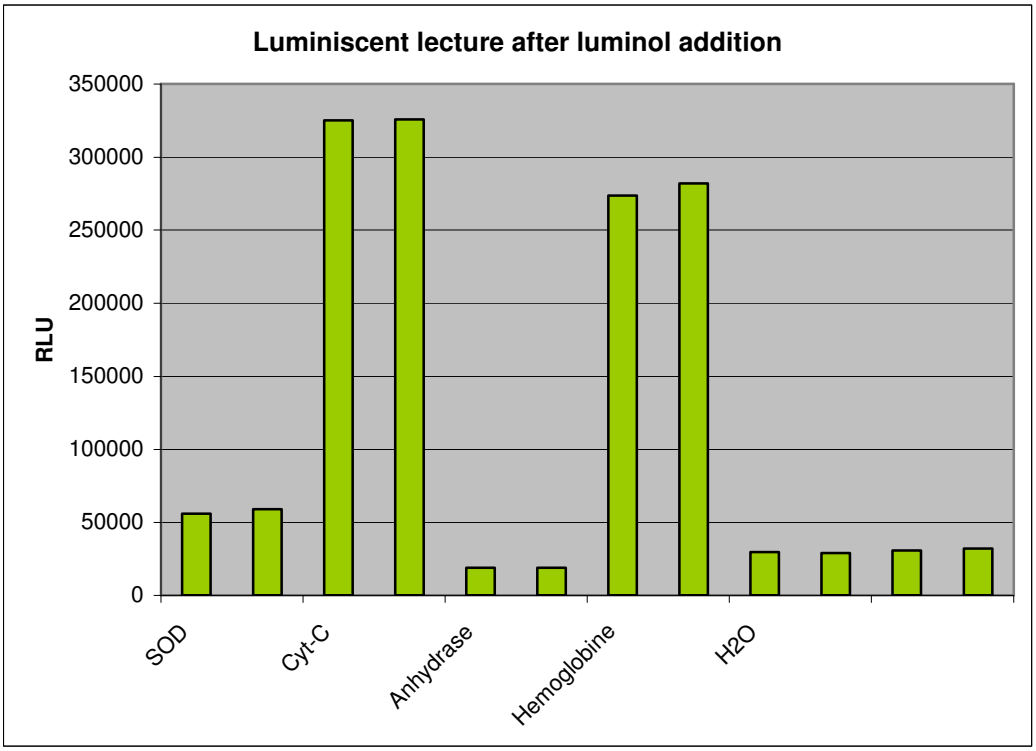


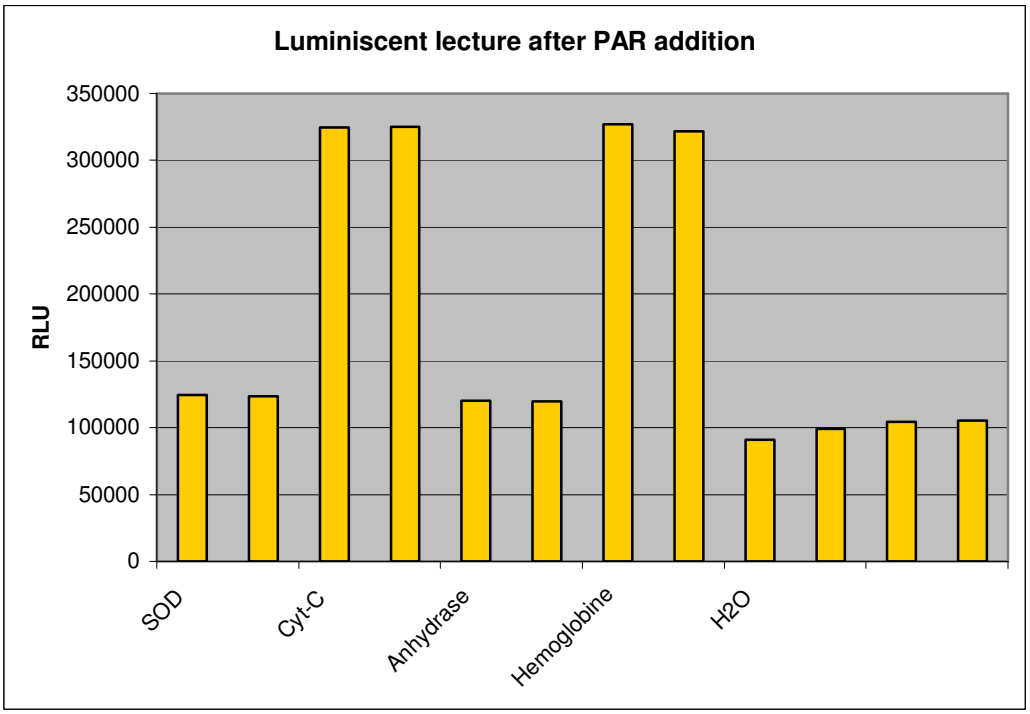


Graph 1: Results showing the relative luminescent units (RLU) for each metal after the luminol and PAR addition. Iron and Cobalt are chemiluminescent in both cases; meanwhile cupper chemiluminescence is dramatically reduced after PAR addition. Manganese which is negative, present luminescence after add PAR. Nickel is negative in both cases.

Table 1: Results obtained for each metallic salt. The RLU and absorbance obtained has been substituted as positive or negative for each test. Positive indicates chemiluminescence for the luminol assays or reddish color in the PAR colorimetric assay.

Metallic salt	Luminol luminescence	PAR luminescence	PAR colorimetric
Fe	+	+	-
Cu	+	-	+
Co	+	+	+
Mn	-	+	+
Ni	-	-	+

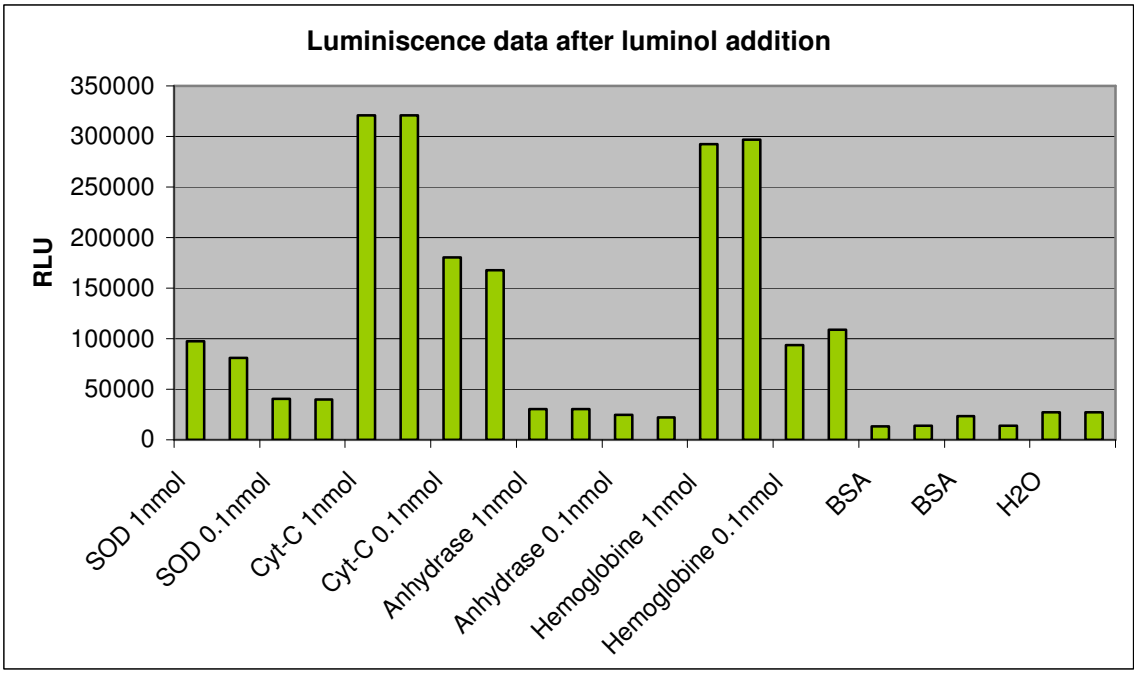


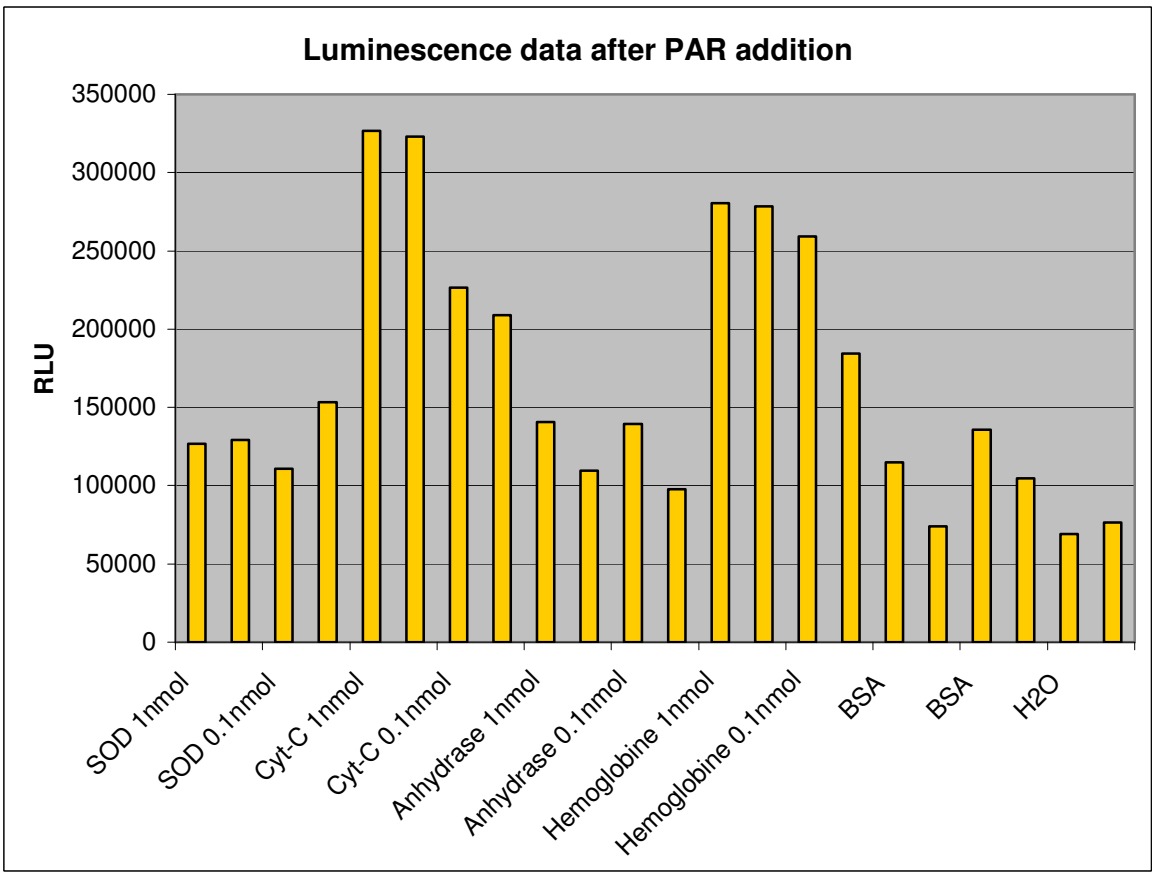


Graph 2: Results showing the relative luminescent units (RLU) for each protein after the luminol and PAR addition. Cytocrome-c (cyt-c) and hemoglobine are luminescent in both cases Superoxide dismutase, (SOD) reduces the luminescence after PAR addition and anhydrase is negative for both cases

Table 3: The RLU and absorbance obtained has been substituted as positive or negative for each test. Positive indicates chemiluminescence for the luminescent assay or reddish color in the colorimetric assay. Comparing these results with the metal salts is possible to deduce presence of metal and the approximate type of metal ion. In the case of the carbonic anhydrase is impossible to differentiate between nickel or zinc using this method, because both metals have the same results.

Protein	**Luminol luminescence**	**PAR luminescence**	**PAR colorimetric**	**Estimated metal**
SOD	+	-	+	Cu
Anhydrase	-	-	+	Ni or Zn
Cytochrome-c	+	+	-	Fe
Hemoglobin	+	+	-	Fe

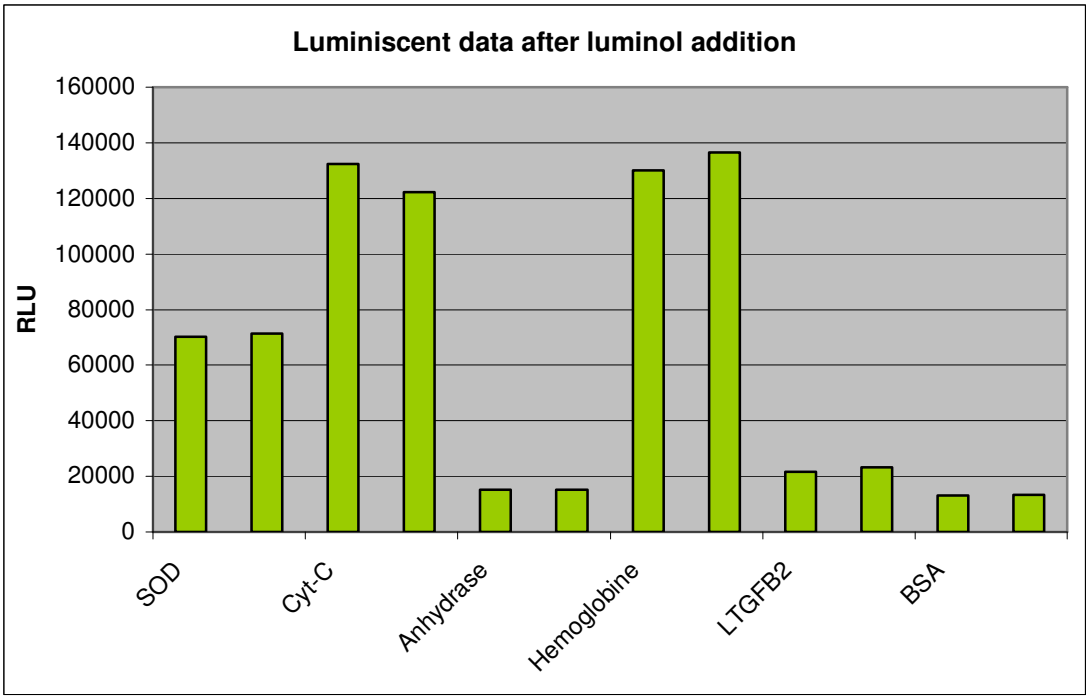


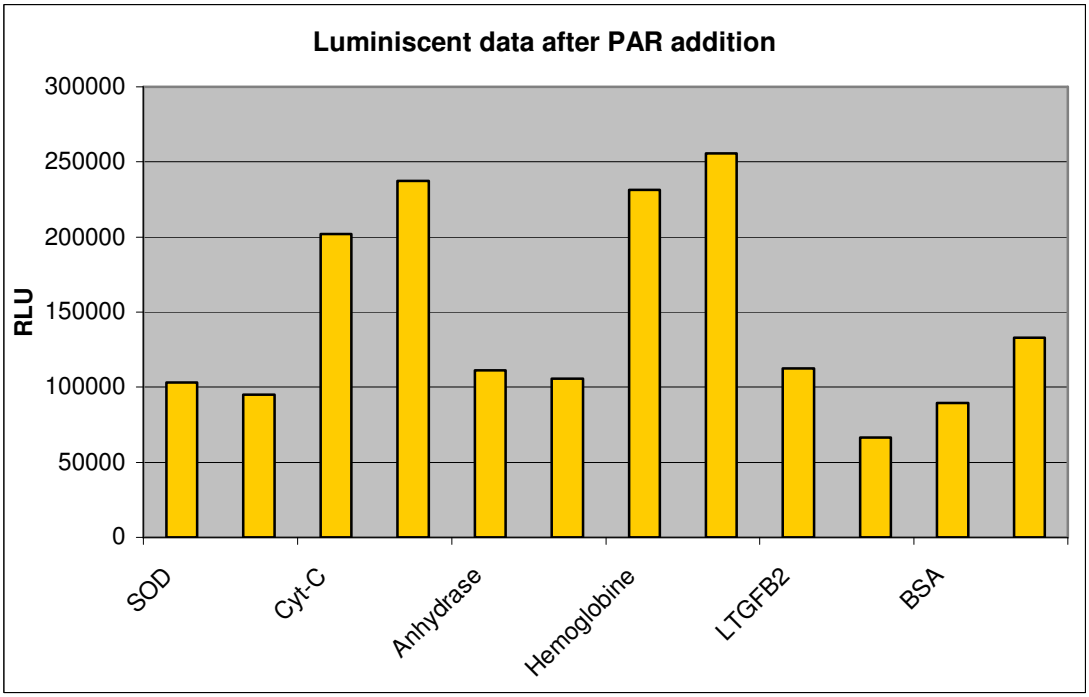


Graph 3: Results showing the relative luminescent units (RLU) for each protein, with two different concentrations of metal ions, 1 and 0.1 nmol. Both concentrations showed enough luminescence to determine the presence of a metal, but 0.1nmol do not give enough robust results to ensure the presence of a metal.

Table 3: The RLU and absorbance obtained has been substituted as positive or negative for each test. Positive indicates chemiluminescence for the luminol assays or reddish color in the colorimetric PAR assay. For the colorimetric assay 0.1nmol of metal is not enough to determine the presence of metal ions, and in some cases could give a ambiguous signal in the chemiluminescent test.

Protein	**Luminol luminescence**	**PAR luminescence**	**PAR colorimetric**
SOD 1nmol	+	-	+
SOD 0.1nmol	ambiguous	-	-
Cytochrome-c 1nmol	+	+	-
Cytochrome-c 0.1nmol	+	+	-
Anhydrase 1nmol	-	-	+
Anhydrase 0.1nmol	-	-	-
Hemoglobine 1nmol	+	+	-
Hemoglobine 01nmol	+	+	-





Graph 4: The luminescence of LTGF-β2 was similar to the negative control (BSA) in all cases. This result will exclude the presence of iron, cupper or cobalt.

Table 4: LTGF-β2 not only showed a negative result in the luminescence assay, also in the colorimetric assay which indicates no presence of metal ion. Although, regarding the bioassay results the LTGF-β2 tested is no longer forming a complex or the protein could be degraded which could explain the negative results obtained.

Protein	Luminol luminescence	PAR luminescence	PAR colorimetric
SOD	+	-	+
Cytochrome-c	+	+	-
Anhydrase	-	-	+
Hemoglobin	+	+	-
LTGF-β2	-	-	-